Characterization of a bacterial tannase from *Streptococcus gallolyticus* UCN34 suitable for tannin biodegradation

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Abstract The gene in the locus GALLO_1609 from *Streptococcus gallolyticus* UCN34 was cloned and expressed as an active protein in *Escherichia coli* BL21 (DE3). The protein was named TanSg1 since shows similarity to bacterial tannases previously described. The recombinant strain produced His–tagged TanSg1 which was purified by affinity chromatography. Purified TanSg1 protein showed tannase activity, having a specific activity of 577 U/mg which is 41% higher than the activity of *Lactobacillus plantarum* tannase. Remarkably, TanSg1 displayed optimum catalytic activity at pH 6–8 and 50–70 °C, and showed high stability over a broad range of temperatures. It retained 25% of its relative activity after prolonged incubation at 45 °C. The specific activity of TanSg1 is enhanced by the divalent cation Ca^{2+} and is dramatically reduced by Zn^{2+} and Hg^{2+}. The enzyme was highly specific for gallate and protocuatechuate esters, and showed no catalytic activity against other phenolic esters. The protein TanSg1 hydrolyzes efficiently tannic acid, a complex and polymeric gallotanin, allowing their complete conversion to gallic acid, a potent antioxidant. From its biochemical properties TanSg1 is a tannase with potential industrial interest regarding the biodegradation of tannin waste or its bioconversion into biologically active products.

Keywords Tannase • *Streptococcus* • Hydrolase • Esterase • Gallic acid • Antioxidant

Introduction

Tannins are water soluble phenolic secondary metabolites of higher plants. Tannins are the fourth most abundant plant constituent, after cellulose, hemicellulose, and lignin (Lekha and Lonsane, 1997). Depending on the origin of tannins their chemistry varies widely, having a molar mass ranging from 300 to 3000 Da, although molecules as large as 20,000 Da have been found. High tannin concentrations are found in nearly every part of the plant, such as bark, wood, leaf, fruit, root, and seed (Serrano et al., 2009). Tannins widely occur in common foodstuffs such as tea, strawberry, raspberry, blackberry, grape, mango, cashew nut, hazelnut, walnut and so on (Mingshu et al., 2006).

The ability of tannins to bind to proteins and other molecules causes serious environmental pollution. This implies the need for ecological–friendly degradation methods for tannic compounds. Furthermore, there is a need in the food industry to solve problems related to the binding of tannins to proteins, starch, and some other nutrients in livestock feeds since it not only affects the nutritional quality of the feed but also decreases digestibility (Chávez–González et al., 2012).

Tannins are generally resistant to biodegradation. Though tannins have toxic effects on various organisms, some microorganisms are resistant to tannins and have the ability to degrade them by the action of a tannase enzyme. Tannase, commonly referred as tannin acyl hydrolase (EC 3.1.1.20), is the most studied enzyme in the biodegradation of tannins. Tannase have received more attention because of their broad range of applications (Chávez–González et al., 2012). Tannase is widely used in the leather, pharmaceutical, beverage, and food industries. So far, the main applications of the tannase are instant tea, acorn liquor, as well as gallic acid production from plant materials rich in gallotannins. In the food industry, tannase is used as clarifying agent in juices and flavoured coffee soft

drinks; in addition, tannase helps to reduce the adverse effects of tannins in beverages and foods (Belmares et al., 2004). The main product of tannase is gallic acid. Gallic acid is used in the pharmaceutical industry as an important intermediate compound in the synthesis of trimethoprim. It is used in the chemical industry as a substrate for chemical or enzymatic synthesis of propyl gallate and other gallic acid esters, cosmetics, hair products, adhesives, and lubricants. Gallic acid is used also in fabrication of semiconductors, dyes and in photographic revelation. Several studies have found that gallic acid and related compounds have important therapeutic properties (Aguilar et al., 2001, 2007).

Although several fungal tannases have been studied and characterized so far, the diversity of applications and conditions in which these enzymes must work also require a large number of different enzymes capable of acting in each condition. Exploration of microbial diversity may help to find new enzymes with interesting properties (Aguilar et al., 2007; Chávez–González et al., 2012).

While many tannases from different fungi have been studied, at present, very little is known about these enzymes in bacteria. Although several sequences from the bacteria, which probably code for different tannases, have been annotated in the databases, there have been very few studies about them (Banerjee et al., 2012). Indeed such studies only concern on three bacterial tannases. A tannase from *Staphylococcus lugdunensis* was genetically identified but was not biochemically characterized (Noguchi et al., 2007). A truncated–tannase from *Enterobacter* sp. has been overexpressed in *Escherichia coli* and its optimal temperature and pH were determined (Sharma and John, 2011). Finally, *Lactobacillus plantarum* tannase is the only bacterial tannase that has been biochemically characterized so far, and its substrate specificity determined (Curiel et al., 2009).

In the present study, we report the cloning the gene encoding TanSg1 tannase from *Sptreptococcus gallolyticus* UCN34 was cloned and expressed in *E. coli*. The production,

purification, and biochemical characterization of the recombinant TanSg1 enzyme are also described.

Materials and methods

Bacterial strains and growth conditions

S. gallolyticus UCN34 (CIP 110142) used through this study was kindly provided by Dr. Philippe Glaser (Institut Pasteur, France). *Escherichia coli* DH10B and *E. coli* BL21 (DE3) were used as transformation and expression hosts in the pURI3–Cter vector (Curiel et al., 2011). The *S. gallolyticus* strain was grown in BHI medium at 37 °C under static condition, and the *E. coli* strains were cultured in Luria–Bertani (LB) medium at 37 °C and shaking at 200 rpm.

PCR amplification and cloning of *Streptococcus gallolyticus* GALLO_1609 gene

Standard molecular biology techniques were performed as described by Sambrook et al. (1989). Chromosomal DNA was extracted from *S. gallolyticus* UCN34. The gene encoding for a putative tannase (*GALLO_1609*, or *tanSg1*) in *S. gallolyticus* UCN34 was PCR-amplified by Prime Star HS DNA polymerase (TaKaRa) by using the primers 774 (5´–*TAACTTTAAGAAGGAGATATACATatg*tcgattaatcaatggatttttg) and 775 (5´–*GCTATTAATGATGATGATGATGATGATG*aacaatggcatccacccattg) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the GALLO_1609 gene sequence are written in lowercase letters). The amplification was for 30 cycles with the following conditions: 95 °C 10 s, 55 °C 5 s, 72 °C for 1:30 min. The

1.4-kb purified PCR product was inserted into the pURI3-Cter vector using a restriction enzyme- and ligation-free cloning strategy (Curiel et al., 2011). The vector produce recombinant proteins having a six-histidine affinity tag in their C-termini. *E. coli* DH10B chemically competent cells were transformed, recombinant plasmids were isolated, and those containing the correct insert were identified by restriction enzyme analysis, and verified by DNA sequencing.

The comparison of DNA and protein sequences, as well as the conceptual translation of the DNA sequence of the *tanSg1* gene, was carried out with the Basic Local Alignment Search Tool (BLAST) program in the NCBI database. Protein sequence alignments were performed using the Clustal W2 program in EMBL-EBI, and protein analysis was carried out in ExPASy (Swiss Institute of Bioinformatics).

Protein expression and purification of recombinant TanSg1 (GALLO_1609) tannase

Protein expression of the *tanSg1* gene was made using *E. coli* BL21 (DE3) cells as host strain. Cells carrying the recombinant plasmid, pURI3-Cter-TanSg1 were grown at 37 ºC in LB media containing ampicillin (100 μg/ml) until an optical density at 600 nm of 0.4 was reached and then induced by adding isopropyl-β-D-thiogalactoside (IPTG) at 0.4 mM final concentration. Following induction, the cells were grown at 22 ºC for 20 h and collected by centrifugation (8,000 *g*, 15 min, 4 ºC). The cells were resuspended in phosphate buffer (50 mM, pH 6.5). Crude extracts were prepared by French press lysis of the cell suspension (three times at 1,100 psi). The insoluble fraction of the lysate was removed by centrifugation at 47,000 *g* for 30 min at 4 ºC, and the supernatant was filtered through a 0.2 μm pore-size filter and then applied to a Talon Superflow resin (Clontech) equilibrated in phosphate buffer (50 mM, pH 6.5) containing 3 M NaCl and 10 mM

imidazole to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted using 150 mM imidazole in the same buffer. The purity of the enzyme was determined by SDS–PAGE in Tris–glycine buffer. Fractions containing the His6–tagged protein were pooled and analyzed for tannase activity.

Enzyme activity

Colorimetric rhodanine assay for tannase activity

Tannase activity was determined using a rhodanine assay specific for gallic acid (Inoue and Hagerman, 1988). Rhodanine reacts only with gallic acid and not with galloyl esters or other phenolics. Gallic acid analysis in the reactions was determined using the following assay. Tannase enzyme (100 µg) in 700 µl of 50 mM phosphate buffer pH 6.5 was incubated with 40 µl of 25 mM methyl gallate (1 mM final concentration) during 5 min at 37 ºC. After this incubation, 150 µl of a methanolic rhodanine solution (0.667% w/v rhodanine in 100% methanol) was added to the mixture. After 5 min incubation at 30 ºC, 100 µl of 500 mM KOH was added. After an additional incubation of 5–10 min, the absorbance at 520 nm was measured on a spectrophotometer. A standard curve using gallic acid concentration ranging from 0.125 to 1mM was prepared. One unit of tannase activity was defined as the amount of enzyme required to release 1 µmol of gallic acid per minute under standard reaction conditions.

Determination of pH and temperature profile of TanSg1

The effects of pH and temperature on the tannase activity of TanSg1 were studied by using buffers of different pH ranging from 3.0 to 10.0. The buffers (100 mM) used were acetic acid–sodium acetate (pH 3.0–5.0), citric acid–sodium citrate (pH 6), sodium phosphate (pH 7), Tris–HCl (pH 8), glycine–NaOH (pH 9), and sodium carbonate–bicarbonate (pH 10). The rhodanine assay was used for the optimal pH characterization of tannase. Since the rhodanine–gallic acid complex forms only in basic conditions, after the completion of the enzymatic degradation of methyl gallate, KOH was added to the reaction mixture to ensure that the same pH value (pH 11) was achieved in all samples assayed.

The optimal temperature was assayed by incubating the purified TanSg1 in 50 mM phosphate buffer (pH 6.5) at different temperatures (4, 22, 30, 37, 45, 55 and 65 °C). For temperature stability measurements, the recombinant tannase was incubated in 50 mM phosphate buffer pH 6.5 at 22, 30, 37, 45, 55 and 65 °C for 30 min and 2, 4, 6, and 18 h. Aliquots were withdrawn at these incubation times to test the remaining activity at standard conditions. The non–incubated enzyme was considered as control (100%).

Effect of additives on TanSg1

The effect of chemical inhibitors and stimulators on TanSg1 activity was investigated by the rhodanine assay using methyl gallate as substrate. The residual tannase activity was measured after the incubation of the purified enzyme with each additive. The additives analyzed were $MgCl_2$, KCl, $CaCl_2$, $HgCl_2$, $ZnCl_2$, Triton–X–100, Urea, Tween 80, EDTA, DMSO, and β–mercaptoethanol. The activity was expressed as a percentage of the activity level in the absence of additives. Tannase activity measured in the absence of any additive was taken as control (100%).

Substrate specificity of TanSg1 analyzed by HPLC-DAD

The substrate specificity of TanSg1 was determined using 17 commercial phenolic esters (methyl gallate, ethyl gallate, propyl gallate, lauryl gallate, methyl benzoate, ethyl benzoate, methyl 4–hydroxybenzoate, ethyl 4–hydroxybenzoate, propyl 4–hydroxybenzoate, butyl 4–hydroxybenzoate, methyl vanillate, methyl 2, 4–dihydroxybenzoate, methyl gentisate, methyl salicylate, ethyl 3, 4–dihydroxybenzoate, ferulic methyl ester, and ferulic ethyl ester) as well as a natural hydrolyzable tannin (tannic acid)

The standard enzyme assay was modified by using 100 µg of TanSg1, 1 mM substrate, and 1 mM $CaCl_2$ in the reaction mixture. As controls, phosphate buffer containing the reagents but lacking the enzyme were incubated in the same conditions. The reaction products were extracted twice with ethyl acetate (Lab–Scan, Ireland) and analyzed hy HPLC–DAD. A Thermo (Thermo Electron Corporation, Waltham, Massachussetts, USA) chromatograph equipped with a P400 SpectraSystem pump, and AS3000 autosampler, and a UV6000LP photodiode array detector were used. A gradient of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied to a reversed–phase Nova–pack C_{18} (25 cm x 4.0 mm i.d.) 4.6µm particle size, cartridge at room temperature as follows: 0–55 min, 80% B linear, 1.1 ml/min; 55–57 min, 90% B linear, 1.2 ml/min; 57–70 min, 90% B isocratic, 1.2 ml/min; 70–80 min, 95% B linear, 1.2 ml/min; 80–90 min, 100% linear, 1.2 ml/min; 100–120 min, washing 1.0 ml/min, and reequilibration of the column under initial gradient conditions. Detection was performed by scanning from 220 to 380 nm. Samples were injected onto the cartridge after being filtered through a 0.45 µm PVDF filter. The identification of

degradation compounds was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers

Results

Sequence comparison of TanSg1 with bacterial tannases

Osawa and Walsh (1993) demonstrated that *S. gallolyticus* strains produce an enzyme, tannase, which hydrolyzes tannins to release gallic acid. In the *S. gallolyticus* UCN34 complete genome, the TanSg1 (GALLO_1609, GenBank accession YP_003431024) protein was annotated as tannase. The DNA sequence was predicted to encode a 470 amino acid sequence protein. Calculated from the amino acid sequence, the molecular mass is 52.98 kDa and the isoelectric point (p*I*) is 5.09. The BLAST analytical program was used to compare the TanSg1 protein from *S. gallolyticus* UCN34 with those deposited in the database.TanSg1 protein is only 29% and 32% identical to TanA from *S. lugdunensis* (GenBank accession BAF03594) and TanLp1 from *L. plantarum* (GenBank accession BAH20446), respectively, two of the three bacterial tannases genetically characterized so far (Fig.1). It should be noted that TanA and TanLp1 are only 27% identical among them. The third bacterial tannase genetically identified, a protein from *Enterobacter* sp. KPJ03, is only 12, 7 and 4 % identical to TanLp1 from *L. plantarum*, TanA from *S. lugdunensis*, and TanSg1 from *S. gallolyticus*, respectively. Protein searches on the databases have revealed that this protein from *Enterobacter* is similar (74% identical) to a central region of proteins from *Klebsiella* KTE92 and *Pantoea* sp. strain AT–9b, among others (data not shown). As compared to *Klebsiella* and *Pantoea* proteins,

the 308–amino acid residues protein from *Enterobacter* lacks an N–terminal region of 136 residues and a C–terminal region of 157 residues.

In spite of the low identity displayed by TanSg1 with TanA and TanLp1, this protein was selected for further study of tannase activity. The comparison of amino acid sequence of TanSg1 with TanLp1, whose tridimensional structure have been recently solved (Ren et al., 2013), revealed that residues important for activity are conserved. TanSg1 possess the conserved motif Gly_{150}–X–Ser–X–Gly_{154} typical of serine hydrolases. The catalytic triad identified in the TanLp1 structure is conserved in TanSg1 (Ser_{152}, Asp_{420}, and His_{452}) as well as the residues which contact with the three hydroxyl groups of gallic acid (Lys_{328}, Glu_{342}, and Asp_{422}) (Fig. 1). Therefore, structural data suggest that TanSg1 could be an active tannase.

Production and characterization of purified recombinant TanSg1

The *tanSg1* gene was cloned into the pURI3–Cter expression vector by a ligation –free cloning strategy described previously (Curiel et al., 2011). The vector incorporates the DNA sequence encoding hexa–histidine to create His–tagged fusion enzyme for further purification step. The integrity of the construct was confirmed by DNA sequencing.

The *tanSg1* gene was expressed in *E. coli* under the control of an IPTG inducible promoter. Cell extracts were used to detect the presence of overproduced proteins by SDS–PAGE analysis. Whereas control cells containing the pURI3–Cter vector did not show protein overexpression, an overproduced protein with an apparent molecular mass around 53 kDa was apparent with cells harbouring pURI3–Cter–TanSg1 (Fig. 2). Since the cloning strategy would yield a His–tagged protein variant, *S. gallolyticus* pURI3–Cter–TanSg1 could be purified on an immobilized metal affinity chromatography (IMAC) resin. The

recombinant protein was eluted from the resin at 150 mM imidazole, and observed as single band on 10% SDS–PAGE (Fig. 2). The enzyme was produced soluble after induction with IPTG for 18 h. Routinely about 12 mg of purified protein from 1–liter culture was obtained.

The TanSg1 protein purified by the affinity resin was biochemically characterized. Since tannase catalyzes the hydrolysis of the galloyl ester linkage liberating gallic acid, the activity of tannase could be measured by estimating the gallic acid formed due to enzyme action (Mueller–Harvey, 2001). A specific method for the detection of gallic acid could be used for a reliable quantification of tannase activity. Inoue and Hagerman (1988) described a rhodanine assay for determining free gallic acid. Rhodanine reacts only with gallic acid and not with galloyl esters or other phenolics. Rhodanine reacts with the vicinal hydroxyl groups of gallic acid to give a red complex with a maximum absorbance at 520 nm. Using methyl gallate as substrate, the specific activity of TanSg1 purified enzyme was 577 U/mg.

The optimum pH for the recombinant TanSg1 enzyme was measured at 30 ºC in 50 mM phosphate buffer at different pH values. The enzyme was active between pH 4 and 9, with an optimal pH around 7, being also highly active at pH 6–8 (Fig. 3A). At pH 5, TanSg1 and TanL1 showed less than 20% activity. At pH 3 and 10 the enzyme completely lost its activity. The optimum temperature for the recombinant enzyme (Fig. 3B) was determined in 50 mM phosphate buffer at pH 6.5. The protein was active between 4 and 65 ºC, with 45 ºC as the optimum temperature, while 60 and 90% of the maximal activity was showed at 4 and 65 ºC, respectively. At any of the temperatures assayed the enzyme showed less than 50% of the maximal activity. Regarding stability, the protein dramatically lost its activity after 30 min at 65 ºC (Fig. 3C). However, the enzyme kept more than 70% activity after 30 min incubation at 55 ºC. Surprinsigly, the enzyme showed

about 50 and 25 % of the maximal activity after 6 and 18 h incubation at 45 ºC, respectively (Fig. 3C).

The enzymatic activity of TanSg1 was tested in the presence of various metal ions and additives (Table 1). TanSg1 was activated by Ca^{2+}. DMSO and Tween–80 also activated TanSg1 protein. Enzyme activity was moderately inhibited by EDTA, Triton–X–100, K^{+}, and Mg^{2+}, and strongly inhibited by urea, β–mercaptoethanol, Zn^{2+}, and Hg^{2+}.

Substrate specificity of TanSg1 tannase

In order to known the substrate specificity of TanSg1, esters from different phenolic acids were assayed. The reaction products released by TanSg1 action were analyzed by HPLC–DAD. As shown in Fig. 4, from the phenolic esters assayed, TanSg1 hydrolyzed only esters from protocatechuic acid (3, 4–dihydroxybenzoic acid) and gallic acid (3, 4, 5–trihydroxybenzoic acid). It is noteworthy to mention that only esters with a short aliphatic alcohol were effectively hydrolyzed. Ethyl (C1) and methyl (C2) gallate were efficiently hydrolyzed, whereas propyl gallate (C3) was only minimally hydrolyzed, and lauryl gallate (C12) was noy hydrolyzed by TanSg1 (Fig. 4).

A hydrolysis experiment with a complex and natural tannin was carried out to determine the hydrolytic activity of TanSg1. Tannic acid, almost exclusively formed by poly–galloyl glucose derivatives, was used as natural tannin to assay TanSg1 activity. The HPLC analysis of the reaction products shown in Fig. 5 verified that TanSg1 is a true tannase. Tannic acid was fully hydrolyzed by TanSg1, and gallic acid was identified as the final product resulting from the degradation (Fig. 5). The extent of autohydrolysis without the enzyme was minimal under the conditions used (Fig. 5).

Discussion

Tannase has been the subject of many studies due to its commercial importance and complexity as catalytic molecule (Chávez–González et al., 2012). Tannases are capable of hydrolyzing complex tannins, which represent the main chemical group of natural antimicrobials occurring in the plants. Tannases catalyse the hydrolysis reaction of the ester bonds present in the gallotannins, complex tannins, and gallic acid esters. The enzyme is used in food and beverage processing; however, the practical use of this enzyme is at present limited due to insufficient knowledge about its properties, optimal expression, and large–scale purification (Aguilar et al., 2007). In the past 20 years, significant progress has been made to improve production processes, including the isolation of new strains of tannase–producing microorganisms, the use of different fermentation systems, and new cost–effective purification methods. The current trend in this field is to apply molecular biology techniques to increase yields and reduce production costs. Tannase is currently commercialized by few companies that sell fungal tannase preparations with different purity and catalytic units depending on the presentation of the product (Chávez–González et al., 2012).

The diversity of applications and conditions in which tannases must work also require a large number of different enzymes capable of acting in each condition. Exploration of microbial diversity may help to find new enzymes with interesting properties (Aguilar et al., 2007; Chávez–González et al., 2012). Proteins encoding putative tannase sequences of 149 bacteria and 36 fungi were retrieved from NCBI dadabase; among them only 77 bacterial and 31 fungal putative tannase sequences were taken which have different amino acid composition (Banerjee et al., 2012). Among the bacterial proteins, a *S. gallolyticus* protein was included. *S. gallolyticus* strains have been isolated as

tannin-resistant bacteria from the feces of different mammalian herbivores, including the koala or the Japanese large wood mouse, and it is also a normal inhabitant of the rumen (Rusniok et al., 2010). Its resistance to tannins is linked to its tannase activity, a characteristics which also led this bacterium to be named "*gallolyticus*" as it is able to decarboxylate gallate, an organic acid derived from tannin degradation (Rusniok et al., 2010). The identification in the *S. gallolyticus* UCN34 genome of a protein, TanSg1, similar to two bacterial tannases genetically characterized, TanA from *Stph. lugdunensis* and TanLp1 from *L. plantarum*, indicated that probably is a functional tannase.

Despite the low amino acid identity (32%) found among TanSg1 and TanLp1 proteins, a carefully examination revealed that residues important for activity are conserved. TanSg1 possess the conserved motif typical of serine hydrolases, the catalytic triad, and the residues which contact with the three hydroxyl groups of gallic acid (Ren et al., 2013). Therefore, structural data also suggested that TanSg1 could be an active tannase.

Multiple sequence alignment of fungal and bacterial tannase protein sequences showed conserved regions at different stretches with maximum homology (Banerjee et al., 2012). Phylogenetic tree showed two different clusters; one has only bacterial tannases and another have both fungi and bacteria showing some relationship between these different groups. TanLp1 from *L. plantarum* is included in the first cluster where only bacterial proteins are included. It has been described that TanLp1 represents a novel family of tannases showing no significant sequence similarity to fungal tannases (Iwamoto et al., 2008; Ren et al., 2013; Banerjee et al., 2012). TanSg1 seems to belong to the same tannase family that TanLp1.

The tannase activity of the recombinant TanSg1 produced in *E. coli* was well demonstrated by its ability to hydrolyze methyl gallate, an ester of the gallic acid. Since

tannase catalyzes the hydrolysis of the galloyl ester linkage liberating gallic acid, the activity of tannase could be measured by estimating the gallic acid formed due to enzyme action (Mueller–Harvey, 2001). Inoue and Hagerman (1988) described a rhodanine assay for determining free gallic acid. Rhodanine assay was used to determine the specific activity of TanSg1, simultaneously, the activity of the previously described TanLp1 was also determined as reference. Using methyl gallate as substrate, the specific activity was 577 U/mg for TanSg1 purified enzyme, 41% higher than that of TanLp1 (408 U/mg). As, the specific activity reported for the truncated tannase from *Enterobacter,* 13.63 U/mg (Sharma and John, 2011), is significantly lower, TanSg1 exhibited the highest specific activity reported so far for a bacterial tannase.

The colorimetric rhodanine assay was used to study the biochemical properties of recombinant TanSg1 tannase. All the fungal tannase studied showed maximum activity at acidic pH values (4.3–6.5), with isoelectric points ranged from 4.3 to 5.1 in most of the cases (Chávez–González et al., 2012). The isoelectric point of TanSg1 is 5.09, similar to fungal tannases; however, the optimum pH is around 7, being also highly active at pH 6–8. Despite TanLp1 and TanSg1 are only 32% identical in their amino acid sequence; they showed an identical pH activity pattern. At pH 5, TanSg1 and TanL1 showed less than 20% activity. The neutral optimum pH of bacterial tannases contrasts with the pH dependence of fungal tannases, which are acidic proteins with an optimum pH around 5.5 (Lekha and Lonsane, 1997).

Most of the tannases have been reported to have optimal temperature of activity between 30 and 40 ºC (Chávez–González et al., 2012). Among bacterial tannases, contrarily to TanLp1 which showed maximum activity at temperatures around 40 ºC without a clear optimum (Curiel et al., 2008), TanSg1 exhibited very high activity at all temperatures assayed, reaching a maximum at 50 ºC. At 4 ºC, TanSg1 still displayed 60%

of the maximum activity. Likewise, 90% of the maximum activity of TanSg1 could be observed at 65 ºC. In addition, TanSg1 kept approximately half of the maximum activity after 6 h incubation at 45 ºC, and 40% after incubation at 22, 30 or 37 ºC during 18 h. The high TanSg1 activity observed within this broad range of temperatures, together with its high specific activity, make this protein the best bacterial tannase candidate for various industrial applications. Note that thermophilicity is related to the capacity of the enzyme to hydrolyze the substrate at high temperatures, while thermal stability is defined as an enzyme ability to resist thermal unfolding in the absence of its substrate. Enzymes displaying optimum activity and thermal stability at higher temperatures are attractive for biotechnological purposes in various industrial sectors. A series of competitive advantages such as faster reaction rates, decreased viscosity in processing fluids, increased solubility of the substrate, and reduced contamination risk by undesired organisms have been proposed for use of thermostable enzymes in biotechnological processes. TanSg1 characterization demonstrated that the enzyme exhibited high thermal stability under prolonged incubation up to 45 ºC.

It has been described that TanLp1 from *L. plantarum* represents a novel family of tannases showing no significant sequence similarity to fungal tannases (Iwamoto et al., 2008; Ren et al., 2013). However, the reported substrate spectrum of fungal tannases and TanLp1 is similar (Curiel et al., 2009). In order to known the substrate specificity of TanSg1, esters from different phenolic acids were assayed. From the phenolic esters assayed, TanSg1 hydrolyzed only esters from protocatechuic acid (3, 4–dihydroxybenzoic acid) and gallic acid (3, 4, 5–trihydroxybenzoic acid), a behaviour that resembles that of TanLp1. However, contrarily to TanLp1, only esters with a short aliphatic alcohol were effectively hydrolyzed. Whereas TanLp1 was able to hydrolyze esters having an alcohol substituent as long as lauryl, propyl gallate was only minimally hydrolyzed by TanSg1

(Fig. 4). Structural differences among both bacterial tannases will be responsible of the different spatial requirements observed for tannase activity among both enzymes.

Since esters having long aliphatic alcohol chain were not hydrolyzed by TanSg1, complex and natural tannins need to be studied as substrates for tannase activity. Because natural tannin extracts probably contained a range of condensed and hydrolyzable phenolic residues, it is difficult to define the substrate range of the enzyme precisely. For this reason, this study was focused to tannic acid, as a relatively well–defined commercially available hydrolysable tannin preparation. Tannic acid obtained from oak gall nuts from *Q. infectoria* was assayed as a tannin of natural origin. Tannic acid is almost exclusively formed by poly–galloyl glucose derivatives whose nature and complexity vary with the plant source. Cantos et al. (2003) distinguished 32 different phenolic compounds from the acorns of *Quercus* species. All of them were gallic acid derivatives. The differences encountered among these tannic acids can be attributed to the fact that different plant varieties produce different types and quantities of phenolic compounds (Hakkinen and Torronen, 2000). Gallic acid is identified as the final product resulting from the degradation of hydrolysable tannins by TanSg1 action. This is a remarkable result in view of the antioxidant properties of gallic acid. In fact, among hydroxybenzoic acids, gallic acid is the most potent antioxidant, being 1.6–and 3.4–fold more active than protocatechuic and syringic acids, respectively (Ordoudi and Tsimidou, 2006). Therefore, the use of TanSg1 may provide an efficient tool to obtain molecules with valuable activities from the degradation of complex tannins present in agricultural wastes.

The use of tannase from different microbial sources may have benefits for different areas such as food, beverage, cosmetic, and pharmaceutical industries, as well as environmental depollution. A tannase may be efficient on one substrate and not on another. For that, specific tannases are needed for specific needs. The biochemical

characteristics showed by TanSg1 from *S. gallolyticus* suggests that TanSg1 is a very promising enzyme for tannin degradation. TanSg1 possesses the highest specific activity and thermal stability described for a bacterial tannase. These advantages make TanSg1 an adequate candidate for industrial applications.

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Figure legends

Fig. 1. Comparison of amino acid sequences of the bacterial tannases genetically identified so far. (A) Alignment of TanLp1 from *L. plantarum*, TanA from *Stph. lugdunensis*, and TanSg1 from *S. gallolyticus*. The amino acid sequence of the truncated tannase from *Enterobacter* sp. KPJ03 is also included. (B) Alignment of TanLp1 from *L. plantarum* and TanSg1 from *S. gallolyticus*. Multiple alignments were done using the program ClustalW2 after retrieval of sequences from BLAST homology searches. Residues that are identical (*), conserved (:) or semiconserved (.) in all sequences are indicated. Dashes indicated gaps introduced to maximize similarities. The serine hydrolase conserved motif is highlighted in yellow; residues of the catalytic triad identified in the structure of TanLp1 are highlighted in blue; and residues which make contacts with the three hydroxyl groups of gallic acid are highlighted in pink color.

Fig. 2. Purification of recombinant *S. gallolyticus* TanSg1 protein. SDS–PAGE analysis of the expression and purification of the His_6–TanSg1. Analysis of soluble cell extracts of IPTG–induced *E. coli* BL21(DE3) (pURI3–Cter) (1) or *E. coli* BL21(DE3) (pURI3–Cter–TanSg1) (2), flowtrough (3), or fractions eluted after His affinity resin (4–6). The arrow indicated the overproduced and purified protein. The gel was stained with Coomassie blue. Molecular mass markers are located at the left (SDS–PAGE Standards, Bio–Rad).

Fig. 3. Some biochemical properties of recombinant TanSg1 protein. (A) Relative activity of TanSg1 versus temperature. (B) Relative activity versus pH. (C) Thermal stability of TanSg1 after preincubation at 22 °C (filled diamond), 30 °C (filled square), 37 °C (filled triangle), 45 °C (cross), 55 °C (star), and 65 °C (filled circle) in phosphate buffer (50 mM, pH 6.5); at indicated times, aliquots were withdrawn, and analyzed as described in the

Methods section. The experiments were done in triplicate. The mean value and the standard error are shown. The observed maximum activity was defined as 100%.

Fig. 4. Enzymatic activity of recombinant *S. gallolyticus* TanSg1 protein against commercial phenolic esters. Hydrolase activity of purified TanSg1 protein compared with control reactions on which the enzyme was omitted. HPLC chromatograms of TanSg1 (100 μg) incubated in 50 mM phosphate buffer pH 6, 1mM $CaCl_2$, and 1 mM of methyl gallate (A), ethyl gallate (B), propyl gallate (C), ethyl protocatechuate (D). The methyl gallate (MG), ethyl gallate (EG), propyl gallate (PG), ethyl protocatechuate (EP), gallic acid (GA), and protocatechuic acid (PA) detected are indicated. The chromatograms were recorded at 280 nm.

Fig. 5. Tannic acid hydrolysis by TanSg1 activity. Hydrolase activity of purified recombinant TanSg1 protein compared with control reactions on which the enzyme was omitted. HPLC chromatograms of control reaction (A) or **TanSg1 (100 μg)** (B) incubated in 50 mM phosphate buffer pH 6, 1mM $CaCl_2$, and 1 mM of tannic acid. The gallic acid (GA) detected is indicated. The chromatograms were recorded at 280 nm.

Table 1

Table 1. Effect of additives on recombinant *S. gallolyticus* TanSg1 tannase activity

Additions (1 mM)	Relative activity (%)
Control	100
EDTA	80
KCl	69
$HgCl_2$	6
$CaCl_2$	120
$MgCl_2$	65
$ZnCl_2$	10
Triton X 100	78
DMSO	144
Tween 80	119
Urea	35
β-mercaptoethanol	16

Figure 1

A

```
TanLp1          ----------------------------------------------------MSNRLI 6
TanSg1          ----------------------------------------------------MSINQWI 7
TanA            MKKTFISLLSATVILSGCGVGEHQNNNSNHDAKGVNTSNVKIKNYNQASSALQIDNSKWK 60
Enterobacter    ----------------------------------------------------GCGGL 5

TanLp1          FDADWLVPEQVQVAGQAIQYYAARNIQYVQHPVAAIQVLNVFVPAAYLHG---SSVNGYQ 63
TanSg1          FDETNNCYMSLKNVYCAQPKDSELEALHIFVPAVYMTADGTIDRDAVVTN---KNGTIYT 64
TanA            YDSKNNVYYQLNISYVSNPQAKNVEKLGIYVPAAYFKGKKNHNGTYTVTVNDAKKVNGYS 120
Enterobacter    CGSINLSLSNASGCVPAMNGEFTVAATNMGHAGSMMDASWALDPQKRIDF-------AYR 58
                 .       . .    :           :  .   :           :          *

TanLp1          RATAPILMPNTVGGYLPGPADDPQRVTWPTNAGTIQQALKRGYVVVAAGIRGRTTVDKSG 123
TanSg1          SQTVPIIFYNDIGGYAECQP--------AMVTPRNQRYLEDGYVLVSVGARGR----QSQ 112
TanA            ARTAPIVYPVNTPGYAEQSAP------TSYRYSNISKYMKAGFIYVEAGLRGRSMSMGNN 174
Enterobacter    ANHLTAQLTKALAAAYYGQRP---------RYAYFMGCSDGGREALMEAQRYP--DDFNG 107
                    .       .                          . *   :  . *       .

TanLp1          QRVG-------QAPAFIVDMKAAIRYVKYNQGRLPGDANRIITNGTSAGGATSALAGASG 176
TanSg1          NGIG-------KAPAGLVDLKAAVRWLRKHHNDIPGDIEKIISVGTSAGGAMSSLLGSTG 165
TanA            SSNASTKSYETGSPWGVTDLKAAIRYYRFNDSSLPGNSSKIYTFGHSGGGAQSAIAGASG 234
Enterobacter    ISAG--------APAAWFSMQNSFFHGWNVVANLRADGTPILLQNRLALIHQAALAHCPT 159
                   .        :*    .:: :.         : .:   *   .  .    :::  ..

TanLp1          NSAYFEPALTALGAAP-------ATDDIFAVSAYCPIHNLEHADMAYEWQFNGINDWHRY 229
TanSg1          NRAEYLSFLEEIGAELD------QRDDIFAAQCFCPITNLEHADMAYEWMFQAKKIYTFN 219
TanA            DSKLYYKYLEQIGAAMTDKNGKYISDKIDGAMAWCPITSLDQADAAYEWQMGQYGNEGNR 294
Enterobacter    LSGINDGILQNPFACTFS----------RSWIKTCPADGQARSDCLTAEELDVVEKLYQG 209
                       *    *               .    **  .  ::*      :

TanLp1          QP-------------------------VAGTTKNGRPKFEPVSGQLTVEEQALSLALKAQF 265
TanSg1          SR-------------------------VRPQIINKR-------------QQLLSQSLAAEF 242
TanA            KKNSFQKQLSTDLASSYASYLNKLNLKNGNTTLSLTKSKNGQYTEGSYAKYLKKEIEDSA 354
Enterobacter    AR---------------------------------------------------------- 211

TanLp1          STYLN--------QLKLTAS---DGTHLTLNEAGMGSFRDVVRQLLISSAQTAFDQGTDI 314
TanSg1          PEYVN--------SLHLDESLTADGRGGNFYQGILNQLSLSLNKFLAKHAQTNDEKEELA 294
TanA            TEFLNNTTFPYKQNSTEQAGMGNGGPSGGKPSGKMGSMPQMRKQSSNKTYKTMDAYLKDL 414
Enterobacter    ----------------------GSQGEQFAPAGLPIGSELRWPVPATATGSSMSEMMAL 248
                                     ..       . :           .   :

TanLp1          HKYAG----FAVTGNQVTDLDLSAYLKSLT-RMKAVPAFDQLDLTSPENNLFGDATAK-- 367
TanSg1          RELDPQGLWCHFENGQATVFDLDAYVVNYMGRKKDCPAFDSLDYQTPETEVFGNRDKN-- 352
TanA            NKKGT-WITYDKKTKRAHITSLKDFAKYYKQPSKSVSAFDDLKRSQAENEVFGTSGSDSK 473
Enterobacter    PALQY----VLMPGGKQPINKVADFAFNRQNFDRVAALAPLYNATNTNLKPFAAAGGK-- 302
                              :    .:  :        :  .     .   .: : *.    .

TanLp1          -------AKHFTA-----------LAQTRSTVTAQLADAELIQA--------INPLSYLT 401
TanSg1          -------HRHFSENVAKHIEKLPALSDYQKAFQVDLAEEDLILARK-----LLNPMTFLQ 400
TanA            LHFDQSLAKLLTENKSNYSKLNGWNSNYVSSYKNDLTKTDKLGTSMSTRMNMYNPMYYLS 533
Enterobacter    ----------------------------------------------------LDPLAR-- 308
                                                                     :*:

TanLp1          TTSS-----QVAKHWRIRHGAADRDTSFAIPIILAIMLENHG--YGIDFALPWDIPHSGD 454
TanSg1          SDLEEK---QVASHYRICLGAKDADTSFAISYLLALALKKRG--IDVHYELIWGMGHADA 455
TanA            DYYSGYGKSNVANHWRIRTGIQQGDTALNTETNLSLALKERVGSKNVDFKTVWDQGHTMA 593
Enterobacter    ------------------------------------------------------------

TanLp1          YDLG----DLFSWIDGLCQ- 469
TanSg1          DYNE----EFSQWVDAIVH- 470
TanA            ETSGNSDSNFIKWVESINKK 613
Enterobacter    --------------------
```

B

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TanLp1   -MSNRLIFDADWLVPEQVQVAGQAIQYYAARNIQYVQHPVAAIQVLNVFVPAAYLHGSSV 59
TanSg1   MSINQWIFDETNNCYMSLKNVYCAQPKDSELEALHIFVPAVYMTADGTIDRDAVVTNKNG 60
            *: ***       .:: .  *    :  :  ::  *.. : . ..:   * : ...

TanLp1   NGYQRATAPILMPNTVGGYLPGPADDPQRVTWPTNAGTIQQALKRGYVVVAAGIRGRTTV 119
TanSg1   TIYTSQTVPIIFYNDIGGYAECQP--------AMVTPRNQRYLEDGYVLVSVGARGR--- 109
         . *   *.**:: * :***    .        .  :   *: *: ***:*:.* ***

TanLp1   DKSGQRVGQAPAFIVDMKAAIRYVKYNQGRLPGDANRIITNGTSAGGATSALAGASGNSA 179
TanSg1   -QSQNGIGKAPAGLVDLKAAVRWLRKHHNDIPGDIEKIISVGTSAGGAMSSLLGSTGNRA 168
          :* : :*:*** :**:***:*::: ::. :*** ::**: ******* *:* *::** *

TanLp1   YFEPALTALGAAP-ATDDIFAVSAYCPIHNLEHADMAYEWQFNGINDWHRYQPVAGTTKN 238
TanSg1   EYLSFLEEIGAELDQRDDIFAAQCFCPITNLEHADMAYEWMFQAKKIYTFNSRVRPQIIN 228
          : . *  :**     *****...:*** ********** *:. : :   . *     *

TanLp1   GRPKFEPVSGQLTVEEQALSLALKAQFSTYLNQLKLTAS---DGTHLTLNEAGMGSFRDV 295
TanSg1   KR-------------QQLLSQSLAAEFPEYVNSLHLDESLTADGRGGNFYQGILNQLSLS 275
          *             :* ** :* *:*. *:*.*:*  *   **   .: :. :..:

TanLp1   VRQLLISSAQTAFDQGTDIHKYAG----FAVTGNQVTDLDLSAYLKSLT-RMKAVPAFDQ 350
TanSg1   LNKFLAKHAQTNDEKEELARELDPQGLWCHFENGQATVFDLDAYVVNYMGRKKDCPAFDS 335
         :.::* . ***  ::    ::         . ..*.* :**.**: .   * *  ****.

TanLp1   LDLTSPENNLFGDATAKAKHFTA-----------LAQTRSTVTAQLADAELIQA---INP 396
TanSg1   LDYQTPETEVFGNRDKNHRHFSENVAKHIEKLPALSDYQKAFQVDLAEEDLILARKLLNP 395
         **  :**.::**:   : :**:            *:: :.:. .:**: :** *   :**

TanLp1   LSYLTTTSS--QVAKHWRIRHGAADRDTSFAIPIILAIMLENHGYGIDFALPWDIPHSGD 454
TanSg1   MTFLQSDLEEKQVASHYRICLGAKDADTSFAISYLLALALKKRGIDVHYELIWGMGHADA 455
         :::* :  .  ***.*:**  ** * ******. :**: *:::* .:.: * *.: *:.

TanLp1   YDLGDLFSWIDGLCQ 469
TanSg1   DYNEEFSQWVDAIVH 470
             :: .*:*.: :
```

Figure 2

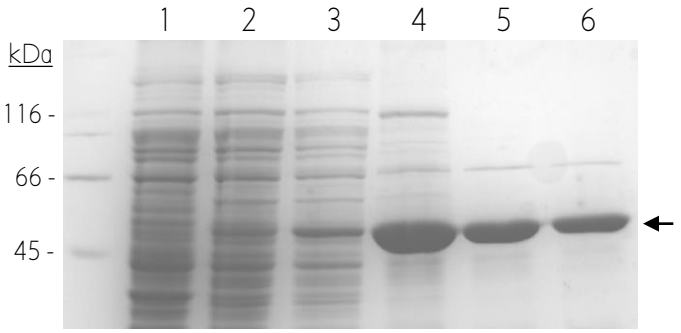
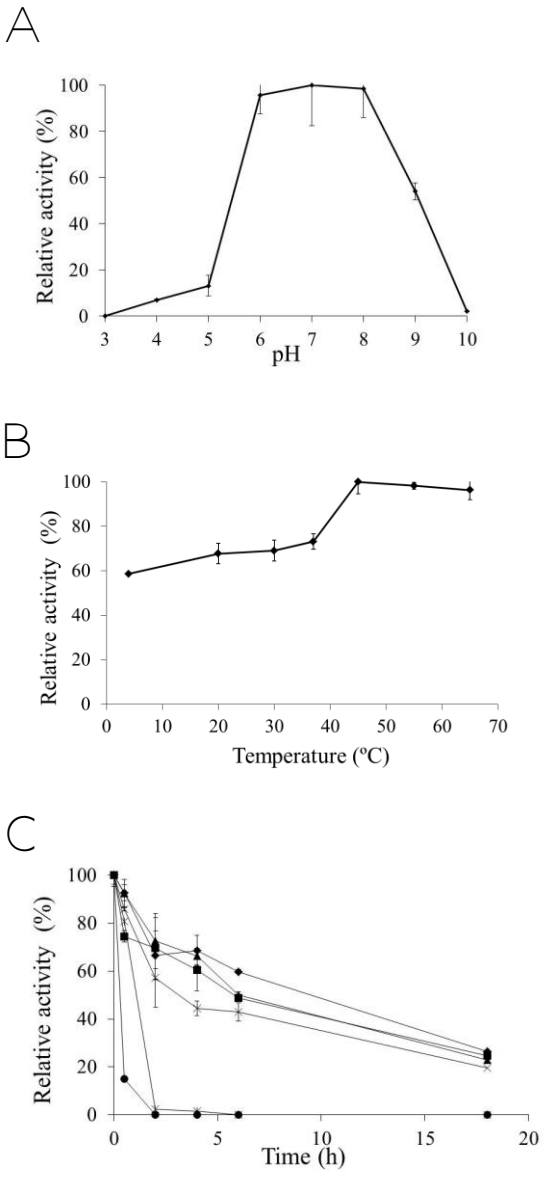


Figure 3


A
Relative activity (%)
100
80
60
40
20
0
3
4
5
6
7
8
9
10
pH
B
Relative activity (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Temperature (ºC)
C
Relative activity (%)
100
80
60
40
20
0
0
5
10
15
20
Time (h)